Intracellular Cytokine FACS-Staining Protocol

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Stimulation

1. Separate PBMC using Ficol-Paque and wash 2.5 times with 10 ml culture medium
2. For prestaining with CD4 and CD62L, suspend cells (at least 2 x 106, but 4 x 106 is better) in 100µl culture medium, add stain, and incubate for 15 min at RT.
   a) Half the cells will be unstimulated: add 50µl of stained cells to 950µl of culture medium in 24 well plate
   b) Half the cells will be stimulated: add 1µl of metalloproteinase inhibitor (1:1000=10µM, stock is 10mM) to the 50µl of remaining cells; mix gently, and incubate a few minutes. In the meantime, to 947µl of culture medium in the 24 well plate, add 1µl PMA (diluted 1µl to 1000 from stock), 1µl ionomycin, 1µl monensin, and mix. If you choose to culture instead in 2ml, then double these amounts. Then add the cells, gently mix using P1000 pipetteman, and incubate at 37°C for 6 hours.
3. For prestaining with γδ reagents, the procedure is the same except that after the staining, the cells must be washed once with large volume (3-5ml). Also, metalloproteinase inhibitor is not used. **Special note:** The unstimulated control cultures should not be prestained with the γδ reagents; instead the γδ reagents should be included with the other surface reagents at the time of staining. (The prestained unstimulated γδ cells lose staining over 6 hrs in culture.)
4. For the cells that will be stained for **perforin**, no stimulation or prestaining is required. Set aside an appropriate number of cells and leave them on ice or at room temp during the 6 hr time period so that all cells can be stained simultaneously.
5. Harvest cells into conical tubes and place them on ice at the end of culture period
6. Pellet cells
7. Wash the cells once with ice-cold PBS/BSA/Azide (2ml)

Surface staining:

8. Suspend cells in ice-cold PBS/BSA/Azide (50µl for each test = 1-2 x 10^6 cells)
9. Transfer cells to 96-well plate containing the surface antibodies (if using EMA to exclude dead cells, include with surface stains, final conc. = 5µg/ml). If using PI (e.g., with perforin), include PI with the surface stains; use 50x, twice the usual concentration.
10. Stain for 15 min on ice in dark
11. Add 150µl PBS and spin. Wash once with 200 µl PBS and spin.
12. Resuspend in 100µl PBS (containing no protein). If using EMA, expose to light for 5 to 10 minutes on ice at this point before fixation.

EMA
10→490 µl
1→20
total
1/1000 dil

Fixation:

13. Add 100µl of 4% Formaldehyde and mix well (final conc. = 2%)
14. Let the cells stand at RT in dark for 20 min
15. Pellet the cells
16. Wash the cells twice with ice-cold PBS/BSA/Azide buffer
17. Pellet the cells and resuspend in 150 µl of permeabilization buffer. Mix gently with multichannel pipette
18. Incubate cells at room temperature for 10 min
19. Pellet cells (aspirate very carefully, or flick out supernatant)

Intracellular staining

20. Resuspend in 25 µl per well of permeabilization buffer containing intracellular Abs (cytokine, perforin)
21. Incubate cells at RT for 30 min in the dark
22. Wash twice (2.5x) with 150-200 µl of permeabilization buffer. (Note that one wash may be sufficient, but more washes may decrease the background).
23. Wash twice with 200 µl of PBS/BSA/Azide buffer (no Saponin)
24. Suspend cells in 200 µl of PBS/BSA/Azide buffer (no Saponin) and transfer to FACS tubes. For HIV samples, this final resuspension should be in staining media (without serum) containing 0.5% paraformaldehyde.
25. FACS analysis

Reagents and Solutions

1) **PMA + Iono**

***Stock soln.**

1mg/ml PMA (DMSO)

2 mM Iono (DMSO)

2) **Monensine and KB 8301 (metalloproteinase inhibitor)**

Stock soln.

2 mM Monensine (ethanol)

10 mM KB 8301 (DMSO), as sent supplied by PharMingen, dissolve 0.5mg powder in 120µl DMSO. Store frozen.

3) **PBS/BSA/Azide buffer**

Mix:	**50 ml**	**10x PBS, pH 7.4**
	450 ml	**Cell culture grade H_2O**
(+)	**0.5 ml**	**1 M Azide**
	500 ml	

Layer 2.5 g of BSA on top of liquid mixture

Allow BSA to dissolve at RT without stirring

Sterile filter the mixture

Store at 4°C

4) **Permeabilization buffer:**

Mix:	**5 ml**	**10% Saponin in PBS**
(+)	**95 ml**	**PBS/BSA/Azide buffer**
	100 ml	

5) **10% Saponin**

Mix:	**5 g**	**Saponin (Sigma)**
	50 ml	**PBS, pH7.4**

Place at 37°C until the saponin has dissolved completely

Sterile filter the mixture (0.22 µ)

Store at 4°C